Regulatory elements in the first intron contribute to transcriptional regulation of the β_3 tubulin gene by 20-hydroxyecdysone in *Drosophila* Kc cells

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ABSTRACT

We have studied the transcriptional regulation of the β_3 tubulin gene by the steroid hormone 20-hydroxyecdysone (20-OH-E) in *Drosophila* Kc cells. A series of hybrid genes with varying tubulin gene lengths driving the bacterial chloramphenicol acetyl transferase (CAT) gene were constructed. The promoter activity was assayed after transient expression in Kc cells, in the presence or absence of 20-OH-E. We find that 0.91Kb upstream from the transcription start site contain one or several hormone independent positive *cis*-acting elements, responsible for the constitutive expression of the β_3 tubulin gene. In the large (4.5 Kb) first intron of this gene, we identified additionnal hormone dependent negative and positive regulatory elements, which can act in both directions and in a position-independence manner. Then, the negative intron element(s), which repress the transcription in the absence of 20-OH-E has characteristics of silencer.

INTRODUCTION

Many eukaryotic genes require *cis* acting regulatory elements for transcription regulation. These elements appear to act as binding sites for trans-acting factors which stimulate (positive regulatory elements) or repress (negative regulatory elements) promoter activity (1). In mammalians, some of them, called Hormone Regulatory Elements (HRE) are steroid receptors binding sites involved in the transcription regulation by steroid hormones. These HRE are palindromic sequences and generally exhibit the properties of inducible promoter enhancers (2)(3).

20-hydroxyecdysone (20-OH-E) is an hormonal steroid of crucial importance for insect development. In *Drosophila*, the 20-OH-E response seems to be similar to that of other steroid response systems, and require cis regulatory elements called Ecdysone Responsive Elements (EcRE). These EcRE are the presumed binding sites of the ecdysone receptor which is not actually completely characterized (4). In fact the mechanism by which 20-OH-E induces transcription is not as well understood as the mammalian steroid mechanisms. The four small heat-shock genes of *Drosophila* represent the most extensive studies of genes which respond to 20-OH-E. Indeed, sequences found in hsp22, hsp23, hsp26 and hsp27 promoters have been shown to be necessary and sufficient for the 20-OH-E induction of these genes (5−8). Promoter sequence analysis of these heat-shock genes and other 20-OH-E regulated genes such as the Sgs4 gene (9) does not reveal a consensus sequence for the binding of the hormone receptor. Furthermore *Drosophila* EcRE binding site sequences only show partial sequence homology when compared to mammalian steroid receptor binding sites sequences.

Kc cultured *Drosophila* cells constitute a very convenient system for studying the molecular mechanism of transcription regulation by 20-OH-E. In these cells, 20-OH-E induces dramatic changes at the morphological level that reflect changes at the gene expression level (10). 20-OH-E regulates in Kc cells the expression of one of the four β tubulin genes (11).The 60C β tubulin gene encodes, after hormonal treatment, for the β_3 subunit (12). Previous work in Kc cells has described the structure of the gene and demonstrated that its regulation is at least, in part, at the level of transcription (13).

In order to study the molecular mechanism of the transcription activation triggered by 20-OH-E in Kc cells, we described in this work, our attempts to map and identify *cis*-acting element(s) that are involved in 20-OH-E regulated expression of the β_3 tubulin gene. We have constructed a series of hybrid genes with varying gene lengths driving the bacterial chloramphenicol acetyl transferase (CAT) gene. In the presence or absence of 20-OH-E, the promoter activity following transfection in Kc cells was assayed.

We show that elements responsible for the constitutive expression lie within 0.22 and 0.91 Kb upstream from the transcription start site. We also show that the sequences necessary for 20-OH-E regulation of the expression of the β_3 tubulin gene are located downstream from the initiation site of the transcription. The first intron particularly includes negative regulatory element(s) (NRE) intervening in the transcription inhibition in the absence of 20-OH-E and positive regulatory element(s) stimulating the promoter activity after hormonal treatment. These *cis*-acting intron elements can act upstream or downstream from the gene and irrespective of their orientation.

MATERIALS AND METHODS

Cell culture and hormone treatment

The *Drosophila* cell line used in this study was derived from the lines established by Echalier and Ohanessian (14). This Kc 167 line was grown at 23°C in D22 medium with 2% foetal calf serum. 20-hydroxyecdysone (SIMES) was added to cell cultures from a 10mM stock in 95% ethanol to a final concentration of 1μM. The hormonal response was checked by observing the morphological modifications of the cells (15).

Construction of CAT fusion genes

To construct chimeric CAT expression constructs, the plasmid pBLCAT3 (16) was used. Fusions between the CAT gene and the β_3 5′ flanking region were performed within the 5′ untranslated region, at the AvaII site in the β_3 tubulin first exon. A 0.36 Kb BamHI-AvaII fragment, a 1.03 Kb PstI-AvaII and a 6.1 Kb EcoRI-AvaII fragment containing respectively 0.22 Kb, 0.91 Kb and 6 Kb of the β_3 5′ flanking region were purified and subcloned in pBLCAT3 to create pT-0.22, pT-0.91 and pT-6. For that, these fragments were respectively subcloned in BamHI-BglII, PstI-BglII and HindIII-BglII sites of pBLCAT3, after conversion of cohesive ends into blunt ends and ligation. In order to obtain pTI-4.5, a 6.25 Kb PstI-BamHI fragment including 0.91 Kb of the 5′ flanking region, the first exon (0.26 Kb) and the beginning of the first intron (1.64 Kb), a 2.8 Kb BamHI fragment including the central part of the first intron and a 0.13 Kb BamHI-PvuII fragment including the 3′ part of the first intron (0.06 Kb) and a part of the second exon (0.07 Kb) were used for the in frame fusion via PstI and BglII sites in pBLCAT3. From this construct, a 3.4 Kb HindIII-XbaI internal intron fragment was removed and the deleted plasmid was ligated after conversion of cohesive ends into blunt ends to create pTI-1.1. To construct pTDI-1.1(+) and (−), a XhoI 1.1 Kb fragment, which contains most of the β_3 first deleted intron, was removed from the previous construct, blunt ended and inserted in both orientations, downstream from the CAT gene into the SmaI site of pT-0.91. This fragment was also subcloned into the SmaI site of pUC18 to obtain pUCI-1.1. Using the HindIII and EcoRI polylinker sites of pUC18, it was removed and inserted in the natural orientation into the HindIII site of pT-0.91, upstream from the 5′ flanking region, to create pTUI-1.1(+). pUCI-1.1 was used to cleave the 1.1 Kb deleted intron fragment into two fragments of 0.6 and 0.5 Kb by using the SalI sites within the intron and the pUC18 polylinker. These 0.6 and 0.5 Kb SalI fragments were inserted downstream from the CAT gene into the SmaI site of pT-0.91, respectively in the natural orientation to create pTDI-0.6(+), and in both orientations to create pTDI-O.5(+) and (−).

Cell transfection and CAT assays

Cells were transfected using the calcium phosphate DNA coprecipitation method (17) with the following additional modifications. Solutions were as described previously (18). Each assay used $5\times10^6-10^7$ cells in 6 ml of medium to which 40 μg of plasmid DNA were added *per* 90 mm petri dish . After an incubation time of 4 hrs, the cells were washed with 6ml of TBS (25 mM Tris HCl pH 7.4, 0.75 mM Na_2HPO_4, 5 mM Kcl, 15 mM NaCl) for 2mn, changed with 6 ml medium and then incubated for 24 hours with or without a physiological concentration of 20-OH-E (1μM). Cells were harvested and crude extracts were prepared by sonication in 0.25 M Tris HCl, pH 7.8. Assays for chloramphenicol acetyl transferase (CAT) activity were performed according to the method of Gorman *et al* (19) with modifications: the protein concentration of the cell extracts was measured and identical protein amounts *per* sample were used for the enzymatic reaction.

In Kc 167 cells, 40 mg of DNA are necessary to obtain a significative CAT or β galactosidase response. Indeed co-transfections with a standard construct containing the E.coli β galactosidase gene, give rise to very low activities which are not interpretable. As a control for the efficiency of transfection, CAT activity of each construct was compared with that of the plasmid pAcβG-CAT (20), which has the 5C actin promoter fused to the CAT gene. The 5C actin gene is well expressed in Kc cells in the absence or presence of 20-OH-E. In both cases this construct generates a very important activity which represents for our experiments, 100% of relative CAT activity, respectively for treated and untreated samples.

RESULTS

5′ flanking region contains *cis* elements responsible for the expression of the β_3 tubulin gene in Kc cells

To identify ecdysone regulatory elements within the 5′ flanking region of the β_3 tubulin gene, different lengths of this region were fused to the coding region of the bacterial chloramphenicol acetyl transferase (CAT) gene. These constructs were transiently transfected into Kc cultured *Drosophila melanogaster* cells and the response to hormone treatment was determined by CAT assay, in untreated and treated cells. Control expression is mediated by the plasmid pAcβG-CAT as described in material and methods.

When transfection is carried out with the construction including 0.22 Kb upstream from the transcription start site (pT-0.22, fig.1A lane a) the same weak expression (<10% of the control expression) was obtained in untreated and treated cells (fig.1B,C). When the 5′ flanking region was extended to 0.91 Kb (pT-0.91, fig.1 lane b) the CAT expression was increased (30%) (fig.1B,C) but remained constitutive *i.e.* in presence or absence of 20-OH-E. In order to identify ecdysone regulatory elements further upstream from the transcription start site, we fused a large 6 Kb flanking region to obtain pT-6 (fig.1A lane c). The expression of this construct was slightly repressed by 20-OH-E and the CAT activity level in untreated cells was similar to the one obtained with the constitutively expressed construct pT-0.91 (fig.1B,C).

These results show that one or several *cis* positive elements are present between 0.22 Kb and 0.91 Kb upstream from the transcription start site, and are only responsible for the constitutive expression of the β_3 tubulin gene in Kc cells. The 5′ flanking region extending from 0.91 over 6 Kb is not able to confer 20-OH-E regulation to the CAT fusion gene. The promoter strength is almost identical in the corresponding constructs.

The first intron is essential for the β_3 tubulin gene regulation by 20-OH-E in Kc cells

Since the experiments described above have not permitted to identify the elements responsible for 20-OH-E regulation in the 5′ flanking region, we hypothesized that these elements should be localized in an other part of the gene. A structural particularity of the β_3 tubulin gene is a first large intron of 4.5 Kb. This intron is not found in the other *Drosophila* β tubulin genes and what is more this gene is the only one to be 20-OH-E regulated. We decided to construct a series of fusion genes containing the first intron.

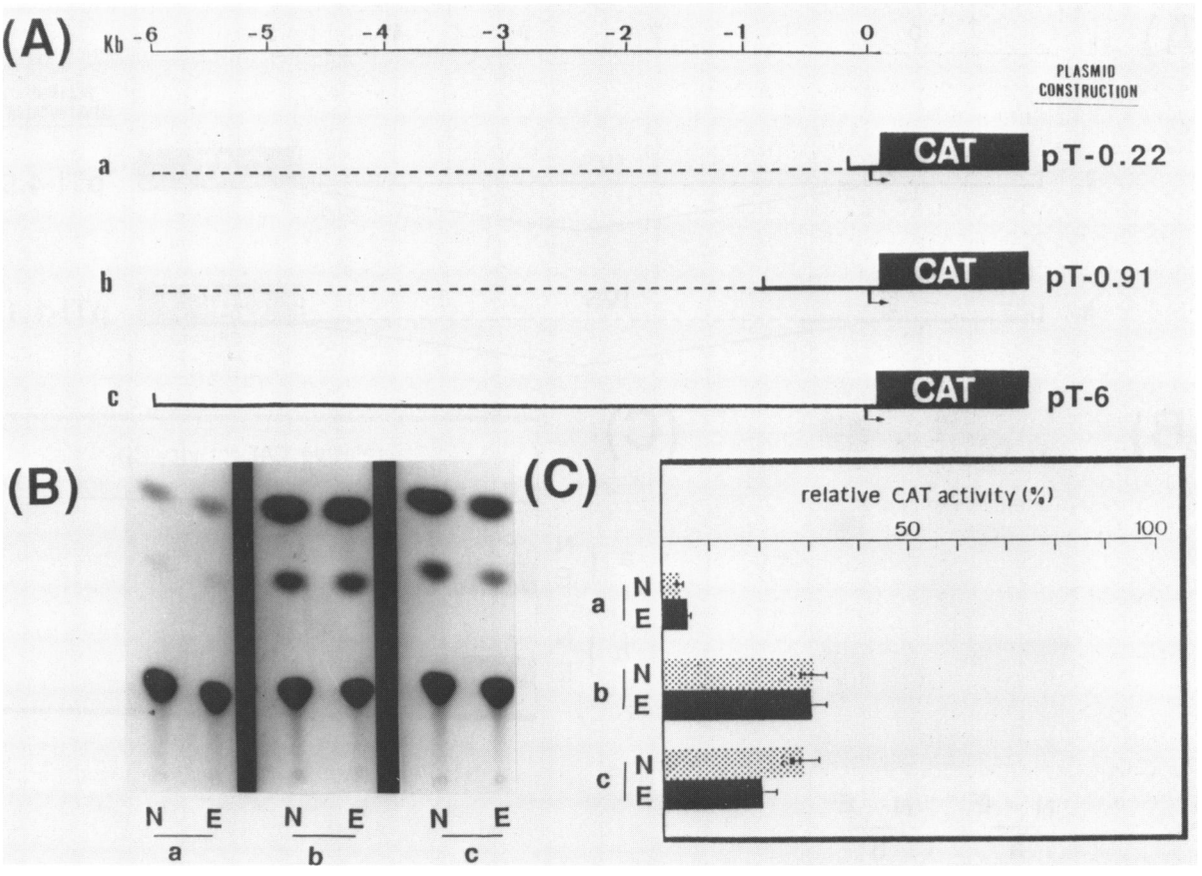

Figure 1: CAT activity of tubulin-CAT fusion gene constructs transfected in Kc cells. A) Shematic representation of plasmids: The bacterial chloramphenicol acetyl transferase (CAT) gene was fused to respectively 0.22 Kb (lane a), 0.91 Kb (lane b) and 6 Kb (lane c) upstream from the start site of the β_3 tubulin gene. (↳) transcription start site. The scale indicates the position of the β_3 tubulin sequences used in the constructs, with respect to the transcription start site. B) Autoradiogram of CAT assays illustrating activities from the same three promoter fusion constructs a) pT-0.22 without (N) and with (E) 20-OH-E. b) pT-0.91 without (N) and with (E) 20-OH-E. c) pT-6 without (N) and with (E) 20-OH-E. C) Relative CAT activity of these three constructs normalized with the plasmid pAcβG-CAT (see mat. and methods). N: without 20-OH-E; E: with 20-OH-E.

pTI-4.5 (fig.2A lane a) contains the same 5′ flanking region as pT-0.91, the first exon, the first large intron and part of the sequence of the second exon. For a correct expression of the CAT gene, it was important to conserve the sequences involved in the splicing process and localized at the 5′ and 3′ sides of the intron. As seen in figure 2B and 2C, in the absence of hormone, pTI-4.5 yielded a weak CAT activity. After 20-OH-E treatment, the expression of this construct including the first intron was strongly induced. The activity varied in treated cells, from 30 to 40% of that produced by pAcβG-CAT.

These results show first that the addition of the first intron to a construct containing 0.91 Kb of 5′ upstream flanking sequences allows the repression of the expression of the β_3 tubulin gene in untreated cells. Moreover, in agreement with the known effect of 20-OH-E on β_3 tubulin gene expression in Kc cells, proper regulation of the gene was recovered with an induction level varying from 4 to 10.

To better define the regions responsible for 20-OH-E regulation, the length of the first intron was reduced. For that, the two unique restriction sites, Hind III and Xba I, were used to delete a fragment of 3.4 Kb. pTI-1.1 (fig.2A lane b) includes the same regions as pTI-4.5 except the deletion of 3.4Kb in the middle of the intron. Splicing sites are conserved at the 5′ and 3′ sides. This construct shows the same features as the previous one, with comparable CAT activities and induction level average as seen in figure 2B and 2C. Thus, a 3.4 Kb internal deletion of the first intron does not affect the expression level and the regulation of the CAT fusion gene.

These results indicate that 1.1 Kb of the β_3 first intron contains sequences acting as a negative regulatory element which mediates the repression of pTI-4.5 and pTI-1.1 expression in the absence of 20-OH-E. This repression was relevied by the addition of the hormone.

However, it is very difficult to compare CAT activities obtained with pT-0.91 in the absence of the intron with pTI-4.5 and pTI-1.1 including the intron. Indeed, the first construct is a transcriptional fusion, without a translated β_3 tubulin region, the second and the third construct, translational fusions with the first exon of the β_3 tubulin gene. We found that adding several exogeneous additional amino-acids to the CAT gene impairs the CAT protein activity (data not shown).

Regulatory sequences within the first intron can either regulate or stimulate the promoter activity in both directions and in a position independence manner

Because many eukaryotic transcriptional regulatory elements function efficiently at considerable distances upstream or downstream from promoters, irrespective of their orientation, we investigated whether the negative regulatory sequences present in the first intron show any of these properties.

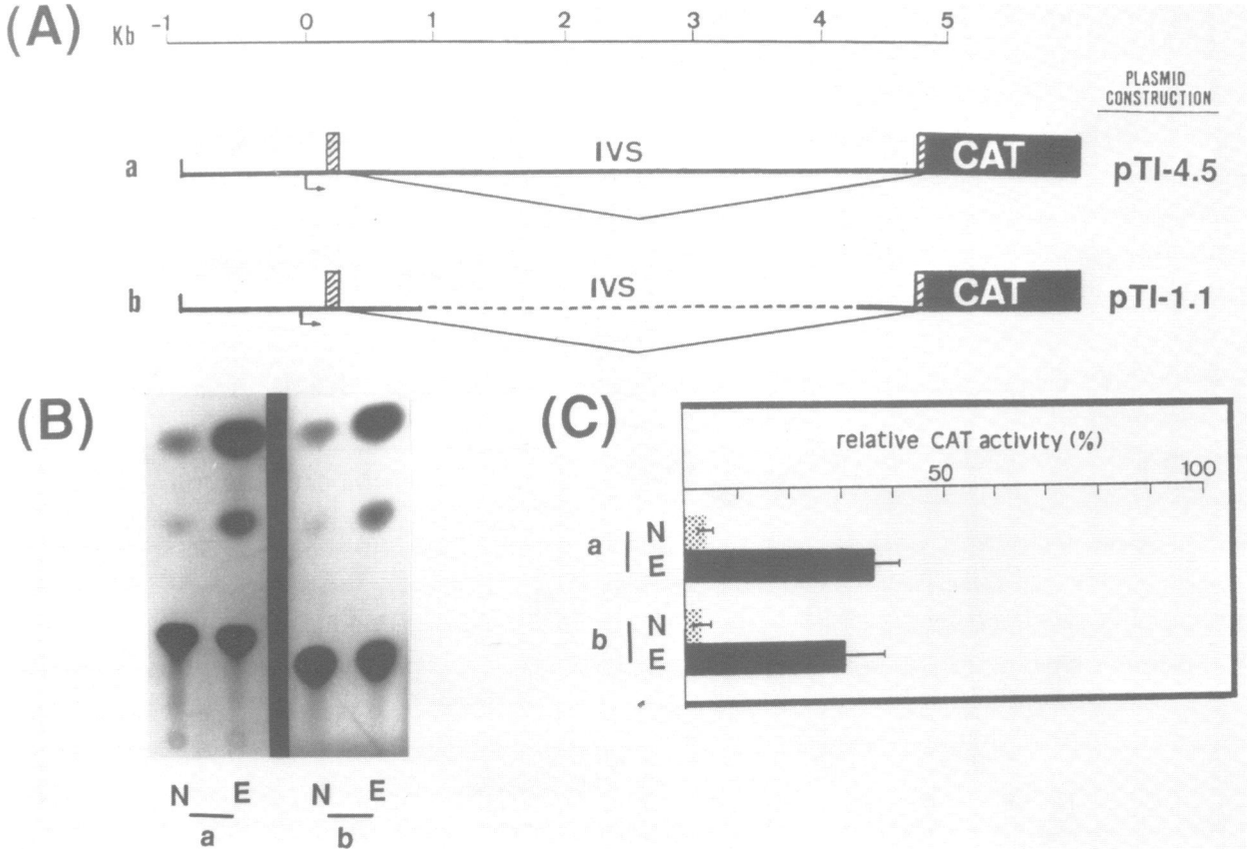

Figure 2 : CAT activity of two tubulin-CAT constructs, including the 5′ flanking region and the first intron of the β_3 tubulin gene. A) The CAT gene was fused to 0.91 Kb upstream from the start site, the first exon and the large first intron (IVS; lane a) or the first deleted intron (lane b) of the β_3 tubulin gene. (↳) transcription start site; hatched boxes: coding sequence of the gene; (---) deleted sequence. B) Autoradigram corresponding to CAT assays from these two constructs: a) pTI-4.5 without (N) or with (E) 20-OH-E. b) pTI-1.1 without (N) or with (E) 20-OH-E. C) Normalized relative CAT activity of pTI-4.5 and pTI-1.1 (see mat. and methods) N: without 20-OH-E, E: with 20-OH-E.

Since the 0.91 Kb flanking region present in pT-0.91, was not efficient at repressing the transcription in the absence of 20-OH-E, we used this construct to test the properties of the intronic sequences. As seen in figure 3A three plasmids were constructed by inserting at various positions the first deleted intron in pT-0.91: pTDI-1.1(+) (lane a) contains the intronic sequences fused in the natural orientation downstream from the CAT gene; pTDI-1.1(−) (lane b) is the same construct with the deleted intron inserted in the opposite orientation; in pTUI-1.1(+) (lane c) the deleted intron is fused in the natural orientation, upstream from the 5′ flanking region. In all the cases, we see that CAT activity is low in untreated cells and high in treated cells (fig.3B,3C) therefore showing that the regulation of the β_3 tubulin gene is present. Indeed with pTDI-1.1(+), the CAT gene regulation leads to an average induction level of 7, which is similar to the previous data, obtained when the full lenght intron is in its physiological position. However, when comparing to this plasmid, pTDI-1.1(−) shows a little higher CAT activity in untreated cells with a lower induction average of about 3. This indicates that the repression is less efficient when the intronic sequences are in the opposite orientation. Analysis of CAT activity level obtained from pTUI-1.1(+) did not reveal significative differences with these of pTDI-1.1(+) although the average level of induction of about 6 is a little lower.

So, in Kc cells, the first intron of the β_3 tubulin gene contains negative regulatory elements which can act in either orientation, and upstream or downstream from the gene, to repress the transcription in absence of 20-OH-E.

Because all the three plasmids are transcriptional fusion, CAT activities can be compared with these of the constitutive expressed construct pT-0.91. It appears that expression levels are greater (about 2 fold more) after hormonal treatment in the presence than in the absence of the intronic sequences. This suggests that the intron is also involved in the transcription activation of the β_3 tubulin gene under 20-OH-E treatment.

A 3′ 0.5 Kb fragment of the first intron is sufficient for 20-OH-E regulation

In order to identify ecdysone regulatory elements responsible for both transcription inhibition without 20-OH-E and transcription activation under hormonal treatment, the first deleted intron of 1.1 Kb was cleaved at a Sal I site into two fragments of 0.6 and 0.5 Kb. Afterwards these fragments were inserted downstream from the CAT gene.

As seen in figure 4A, in the constitutively expressed plasmid pT-0.91 either 0.5 Kb of the intron 3′ side or 0.6 Kb of the 5′ side were inserted downstream from the CAT sequence in the natural orientation: this creates respectively plasmids pTDI-0.5(+) (lane a) and pTDI-0.6(+) (lane c). When cells are transfected with pTDI-0.5(+), transcription inhibition and activation are similar to those observed with pTDI-1.1(+), in the absence or presence of 20-OH-E (fig.4B,4C). When cells

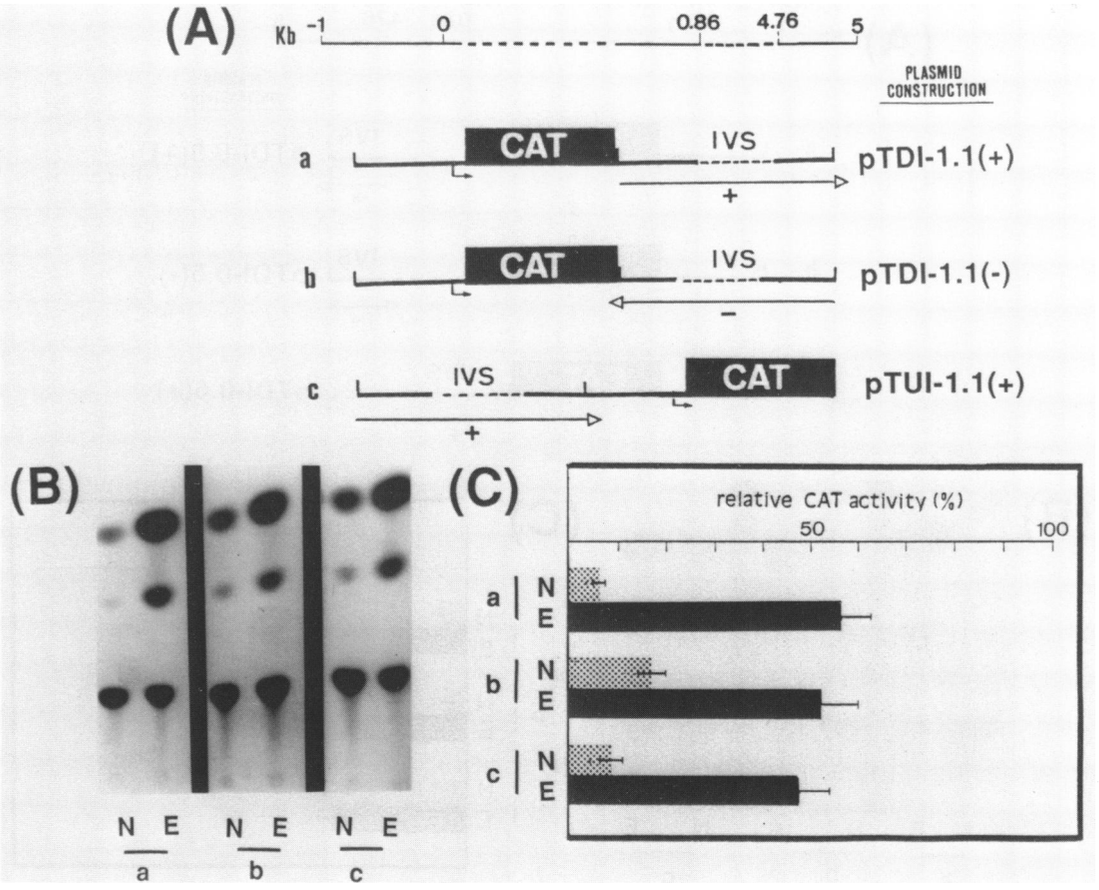

Figure 3: CAT activity of tubulin-CAT fusion genes constructs including 5′ and intron sequences. A) The CAT gene was fused to 0.91 Kb upstream from the start site, and the deleted intron (1.1 Kb) was added respectively downstream from the CAT gene, in positive orientation (lane a); downstream from the CAT gene, in opposite orientation (lane b); upstream form the CAT gene, in positive orientation (lane c). (↳) transcription start site; (---) deleted sequences. B) Autoradiogram corresponding to CAT assays for these three constructs. a) pTDI-1.1(+) without (N) and with (E) 20-OH-E. b) pTDI-1.1(−) without (N) and with (E) 20-OH-E. c) pTUI-1.1(+) without (N) and with (E) 20-OH-E. C) Relative CAT activity of these three tubulin-CAT constructs (see mat. and methods). N: without 20-OH-E, E: with 20-OH-E.

are transfected with pTDI-0.6(+) which contains only the first part of the intron sequence, no difference in CAT activity in untreated and treated cells is detected. Activity levels are similar to these obtain with pT-0.91, which does not contain any first intron sequence.

These results show that negative and positive sequences, which are 20-OH-E dependent, are localized within 0.5 Kb of the intron 3′ side. To verify the action in either orientation of these elements, intronic sequences downstream from the CAT gene of pTDI-0.5(+) were inverted to obtain pTDI-0.5(−) (lane b). With these two constructs, CAT activities are almost equivalent, respectively in untreated and treated cells. Thus, in accordance with the previous results, 20-OH-E dependent elements, present in the 3′ 0.5 Kb fragment of the intron are functional in the positive as well as in the opposite orientation.

DISCUSSION

We have examined the mechanism regulating the transcription of the 20-OH-E inducible, β_3 tubulin gene, in *Drosophila* Kc cells.

Using transient DNA transfer experiments, we have shown that 0.22 kb upstream from the transcription start site leads to a very weak expression of this gene, in the presence or absence of 20-OH-E. Sequence data did not reveal any classical TATA box consensus (TATAAAA) at −30. The sequence found between −28 and −35 (GAACATC) (13) is very similar to that of the β_2 tubulin gene (GAACATT), localised between −26 and −32 (21). The position and the conservation of this sequence are in agreement with these of a TATA-like element. On the other hand, we have also shown that the 0.91 kb region upstream from the transcription start site, contains hormone independent positive element(s), leading to a strong constitutive expression of the β_3 tubulin gene. Indeed, with this part of the 5′ flanking region, the regulation of this gene expression by 20-OH-E is absent. These results are in accordance with sequencing data which do not reveal neither HRE nor EcRE sequences (3)(6) in this region (13). Moreover, even with 6 kb of the 5′ flanking region, the 20-OH-E regulation is absent. The weak repression of the gene expression, observed after hormonal treatment, suggests the presence of 20-OH-E dependent negative regulatory elements far upstream from the start site.

DNA sequences responsible for regulation by steroid hormones, have generally been found in the 5′ flanking regions of genes (22−24). For several years, many reports have described the critical role of introns in the expression of eukaryotic genes, since they may carry transcriptional regulatory elements within their introns (25−28). However, such elements have only been described for a few hormonally regulated genes

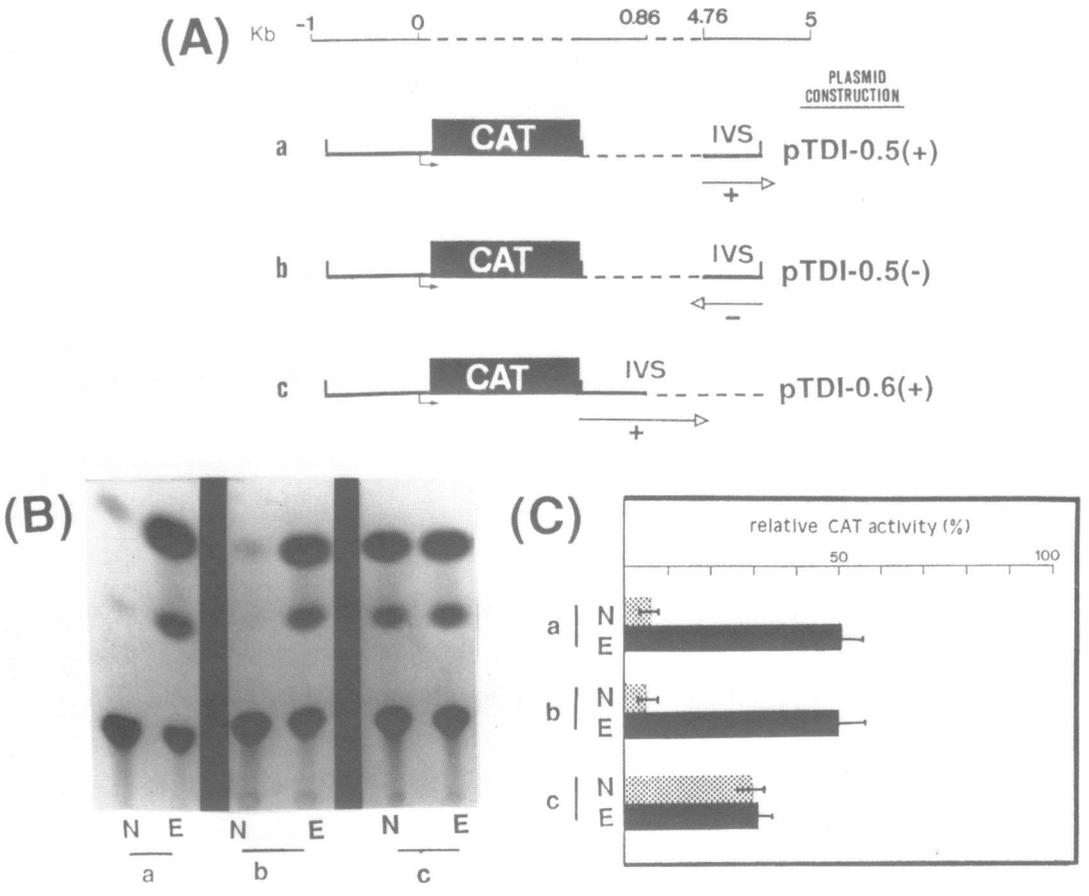

Figure 4 : CAT activity of tubulin-CAT constructs including the 5′ flanking region and respectively the 5′ or 3′ region of the first intron. A) The CAT gene was fused to 0.91 Kb upstream from the start site, and were added: the 3′ 500 bp of the first intron in the positive orientation (lane a); the 3′ 500 bp of the first intron in the opposite orientation (lane b); the 5′ 600 bp of the first intron in positive orientation (lane c). (↳) transcription start site; (---) deleted sequence. B) Autoradiogram corresponding to CAT assays from these three constructs: a) pTDI-0.5(+) without (N) and with (E) 20-OH-E. b) pTDI-0.5(−) without (N) and with (E) 20-OH-E. c) pTDI-0.6(+) without (N) and with (E) 20-OH-E. C) Normalized relative CAT activity of these three constructs (see mat. and methods) N: without 20-OH-E, E: with 20-OH-E.

i.e. uteroglobin gene (29), human (30−31) and rat (32) growth hormone gene. In these two last cases, the intron contains GRE (glucocorticoid response element) and TRE (thyroid response element), respectively. The β_3 tubulin gene belongs to this category. Indeed, addition of the first intron to 0.91 Kb upstream from the start site, allows 20-OH-E regulation. Up to now, the shortest regulated construct includes the 3′ 500 pb fragment of this intron. However, the ability of this fragment to regulate the expression of the gene, does not exclude the presence of additional EcRE in other parts of the intron.

Transcription activation of genes induced by steroid hormones involves binding of the hormone to the receptor, followed by a specific binding to HRE, which exhibit the properties of positive regulatory elements. In some cases, as for the chicken ovalbumin gene, the promoter activity is repressed by negative regulatory elements and the repression is relieved by steroid hormones (22). In the case of the β_3 tubulin gene, the 5′ flanking region contains 20-OH-E independent constitutive element(s), and the first intron includes negative regulatory element(s) (NRE) 20-OH-E dependent. Distantly located eukaryotic *cis*-repressor sequences often work through an indirect mechanism, whereby they interfere with the binding of positive control factors to a neighboring enhancer (33)(34). The β_3 tubulin NRE does not appear to function in this way. It appears to act over a long distance to influence the activity of the basal promoter, similar to the activity of a yeast silencer sequence (35). The exact spacing of the β_3 NRE relative to the transcription start site does not appear to be critical because it can mediate the repression of the activity of a 1Kb promoter by being placed upstream, downstream and in both orientations. Moreover the activity of the NRE in represssing transcription from a basal promoter makes it similar to the activity of a yeast 'silencer' sequence as defined in (35).

Besides the negative effect, the first intron is also involved in the activation of the transcription. Indeed, 20-OH-E does not simply relieve the transcription repression because higher CAT activities are obtained after hormonal treatment, with constructs carrying intron sequences, compared to the constitutive one (see Results).

The model that we propose for the β_3 tubulin gene consists of a repressor-silencer system localized in the first intron and locking the transcription without 20-OH-E. Addition of 20-OH-E would relieve this repression, resulting in activated transcription. This is mediated by positive elements localized in intronic regions and possibly by cooperation between these intronic elements and the 5′ *cis* positive elements. Further studies will allow to precise these different regulatory elements. So, 20-OH-E not only derepresses the transcription of this gene but also activates it. A similar kind of regulation of a hormone

responsive promoter being repressed in abscence of hormone and being derepressed in presence of hormone has been recently described (36)(37). The thyroid-hormone α-receptors (TRα) regulate the expression of several thyroid-hormone-responsive genes in vertebrates by binding to the TREs and functionning as TRE-specific repressors in the abscence of hormone. In the presence of hormone they act as TRE-specific activators. So we can speculate, in the case of the regulation by 20-OH-E of the β_3 tubulin gene in Kc cells, that unfilled 20-OH-E receptors could act as repressors. Although we have not yet proved that the repression mediated by the NRE of the β_3 tubulin gene involved an 20-OH-E receptor, it has been reported that ecdysteroid receptors are permanently located in the nuclei (38). Moreover we can test this hypothesis by purifying the repressor of the β_3 tubulin gene in Kc cells in absence of hormone and looking if it can be a steroid binding protein.

The negative regulation of the β_3 tubulin promoter by a *cis*-acting negative regulatory element (NRE) is probably mediated at the level of DNA-protein and protein-protein interactions. It is not due to a simple steric hindrance mechanism preventing transcription complex from going further downstream this element in absence of hormone like in (39). Moreover these intron sequences could interact with 5′ flanking sequences through DNA binding proteins as described for the Human α1 collagen gene (40). These interactions between distant proteins can be explained by the looping model proposed by Ptashne (41).

During *Drosophila* development, this β_3 tubulin gene is expressed at mid embryogenesis and at the end of the third instar larvae-early pupae (42)(43) and is also 20-OH-E regulated (44). The β_3 tubulin gene is a good marker for mesoderm development (45)(46). Concerning the regulation of this gene in *Drosophila*, Gash *et al* (47) have shown that sequences upstream from the cap site contain distinct *cis*-acting elements, for constitutive and tissue specific expression. In addition, some intron sequences are also involved in tissue specific expression. Although the β_3 tubulin gene sequence is almost identical in Kc cells and in *Drosophila*, the different cellular contexts of these two models involve differences in the requirement of *cis* acting elements used for the regulation of the expression of the gene.

In this paper, we have described *cis*-acting elements, which can act in both directions and in a position-independance manner, involved in 20-OH-E regulated expression of the β_3 tubulin gene in *Drosophila* Kc cells. Precise determination of these *cis*-acting elements and *trans*-acting factors involved in 20-OH-E regulation of the β_3 tubulin gene expression is in progress in our laboratory.

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